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α-Thalassemia Carrier Identification by DNA Analysis in the Screening for Thalassemia

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Differentiation between heterozygous α-thalassemia and several phenotypically resembling alleles at the β-globin gene cluster such as coinherited δ- and β-thalassemia or γδβ-thalassemia is a critical step in genetic counseling. In this paper we report our experience in the identification of the α-thalassemia carrier state using polymerase chain reaction (PCR)-based methods, and the feasibility and simplification of screening for thalassemia using this approach. α-Globin genotype was determined by PCR-based method in 526 adult subjects with reduced mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), normal hemoglobin A_2 and F, and normal serum iron. To verify the reliability of the protocol used, in 68 of these subjects we performed globin chain synthesis analysis and in 101 we determined α-globin genotype by Southern blot analysis. Five hundred twenty-one (99%) of 526 subjects examined were identified as carriers of one or two α-thalassemia alleles. The identification of the α-thalassemia carrier state may be fast and accurate by PCR-based method, avoiding other cumbersome and expensive methods such as globin chain synthesis and Southern blot analysis. Am. J. Hematol. 59:273–278, 1998.

Key words: α-thalassemia carrier; screening; PCR

INTRODUCTION

Different types of thalassemia are usually present in the same population and the most common are β- and α-thalassemia. δ-Thalassemia is rare, but important, because when coinherited in *cis* and in *trans* with β-thalassemia, it produces a reduction of hemoglobin (Hb) A_2 levels and its identification may be difficult. The same hematological phenotype can be produced by the very rare γδβ-thalassemia. The hematological characteristics of double heterozygosity for δ- and β-thalassemia resemble those of the α-thalassemia carrier, but the correct identification of these carriers is critical for genetic counseling. In fact, only double heterozygotes for δ- and β-thalassemia and heterozygotes for γδβ-thalassemia are at risk to produce a thalassemia-major offspring when married to a classical high HbA_2 β-thalassemia carrier.

Identification of α-thalassemia carriers may be achieved by globin chain synthesis analysis and/or by Southern blot analysis. Both procedures are relatively complex, time-consuming, and cumbersome. Moreover, they imply the use of radioactive reagents and for these reasons are not practical and suitable for most laboratories. In the last few years several approaches based on Polymerase Chain Reaction (PCR) have been developed for the detection of the most common deletional and nondeletional types of α-thalassemia [1–7]. Deletion defects are detected using two specific oligonucleotide primers flanking the deletion breakpoint. Amplification occurs only in the presence of the deletion, whereas it does not occur in normal controls because the two oligonucleotides are separated by too great a distance. Detection of common nondeletional forms involves selective amplification with allele-specific oligonucleotide probes or with restriction enzyme digestion, when the mutation creates or abolishes a cleavage site. In Sardinia,

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α-thalassemia is very common; also, double heterozygotes for δ- and β-thalassemia have been described [8–10].

In this article, we report our experience in the identification of α-thalassemia carriers using PCR-based methods and the feasibility and simplification of screening for thalassemia using this approach.

MATERIALS AND METHODS

Subjects

During a screening program for β-thalassemia, a total of 526 adult subjects of Sardinian descent were selected, with the following hematological characteristics: reduced (<79 fl) mean corpuscular volume (MCV) and (<27 pg) mean corpuscular hemoglobin (MCH), normal hemoglobin (Hb) A_2 and F (<3.0% and <1.0%, respectively), and normal iron status [i.e., zinc erythrocyte protoporphyrin (ZnPP)] <30 μg/dl or transferrin saturation higher than 16%).

Methods

Red blood cell indices were determined with an automated electronic cell counter (MAX-M-Coulter, I.L., Milan, Italy) calibrated daily using appropriate controls (5C Coulter Cell Control). HbA_2 and HbF were quantitated by high performance liquid chromatography (HPLC) (Variant, BIO-RAD, Milan, Italy) [11]. For ZnPP determination, we used the hematofluorometer ZP 206 (AVIV, Lakewood, NJ) and for serum iron and total iron binding capacity, we used a colorimetric manual method or the automated iron analyzer Ferrochem II (ESA, Chelmsford, MA). Globin chain synthesis analysis was performed on peripheral blood reticulocytes following the method of Kan et al. [12].

The α-globin genotype was defined by PCR-based methods and in part by Southern blot analysis. DNA was extracted from peripheral blood leukocytes by the salting-out method of Miller et al. [13]. Genomic DNA (0.5—1.0 μg) was amplified in a DNA Thermal Cycler-9600 (Perkin-Elmer, Italia, Monza, Italy). We used the same amplification buffer and thermal-cycling conditions to detect the different α-thalassemia alleles. The amplification buffer had the following composition: 67 mM Tris-Hcl pH 8.8, 1.9 mM $MgCl_2$, 67 μM EDTA, 16.6 mM $(NH_4)_2$ SO_4, 10 mM β-mercaptoethanol, and 170 μg/ml bovine serum albumin. Total volume of the PCR reaction was 100 μl. Twenty-five pM of the specific oligonucleotide primers (Table I), 200 μM d NTP, 10% dimetyl-sulfoxide and two units of Taq polymerase (Perkin Elmer Cetus-Norwalk, CT) were added in the tube. After a denaturation step at 94°C for 10 min, a total of 30 cycles were performed: one min at 94°C, one min at 52°C, one and a half min at 72°C, an additional extension step at 72°C for 10 min was included.

Table I reports the oligonucleotide primers used for the selective amplification of different α-thalassemia alleles and the DNA fragment obtained. In Figure 1, the position of these primers along the α-globin gene cluster are indicated. To detect α2 ATG→ACG and α1 ATG→GTG initiation codon mutations, or the α2 IVS-1 pentanucleotide deletion [14–16], we digested the α2 and α1 genes, amplified using C10 and C3 (α2 gene) or C10 and C2 (α1 gene) oligonucleotide primers, with *Nco*I and *Hph*I restriction enzymes respectively, since the restriction sites for these enzymes are abolished by mutations. Separation of the DNA fragments was performed on 1.2% agarose in TBE (tris-borate-EDTA) and the specific bands were visualized on a UV transilluminator after ethidium bromide staining. Digestion with *Nco*I of normal α2 or α1 globin genes produces two fragments of 1.1 and 0.8 kb or 1.3 and 0.8 kb, respectively. The mutated α2 or α1 globin genes cannot be digested by the *Nco*I restriction enzyme and the fragments obtained correspond to the entire amplification product. The α2 IVS-I pentanucleotide deletion is detected, after *Hph*I digestion, by the presence of a 1.4 kb fragment, compared with the 1.1 and 0.3 kb fragments obtained from the normal allele. Hereafter, the α-thalassemia alleles detected by *Nco*I and *Hph*I restriction enzymes will be denoted as superimposed N or H, respectively.

RESULTS

α-Thalassemia Genotype Identification

At the beginning, to verify the reliability of the protocol used, we performed both globin chain synthesis analysis and α-globin gene analysis by PCR, in 68 subjects with microcytosis and normal HbA_2, HbF and serum iron and Southern blot and PCR analysis of α-globin genes in 101 subjects. In all 68 subjects, the α/β globin chain synthesis ratio was less than 0.9 and an α-thalassemia defect was identified by PCR. Figure 2 shows the correlation between the results of globin chain synthesis and α-globin genotype. The most striking α/β imbalance was found in subjects with the $-\alpha/\alpha^N\alpha$ genotype. All the genotypes defined by PCR were concordant with those defined by Southern blot analysis. Five hundred twenty-one (99%) of 526 subjects examined were identified as being carriers of one or two α-thalassemia alleles. In the remaining subjects, the α/β globin chain synthesis ratio was lower than 0.9, indicating the presence of an α-thalassemia defect. The relative frequencies of the different α-thalassemia genotypes are reported in Table II. A total of 13 different genotypes were detected,

TABLE I. Oligonucleotyde Primers Used to Detect the Different α-Thalassemia Alleles*

	Primer sequence (5′–3′)	Fragments (kb)	References
α_2			
C10	GAT GCA CCC ACT GGA CTC CT	1.9	
C3	CCA TTG TTG GCA CAT TCC GG		
α_1			
C10		2.1	5
C2	CCA TGC TGG CAC GTT TCT GA		2
$\alpha^{3.7}$			
C10		1.9	
C2			
$--^{Med}$			
M			
5′ Med	ACA GTC ACT CCT GAG GCC AGT C	0.65	4
3′ Med	GGA GAA GTA GGT CTT CGT GGC		
N			
5′ Med N	TAC AGC AGA GTG AGT GCT GCA T	1.00	
3′ Med			
$-(\alpha)^{20.5}$			
M			
5′ $(\alpha)^{20.5}$	GGC AAG CTG GTG GTG TTA CAC A	1.18	4
3′ α1	CCA TGC TGG CAC GTT TCT GAG G		
N			
C1	TGG AGG GTG GAG ACG TCC TG	1.07	
3′ α1			
$-\alpha^{4.2}$			
M			
G	CCG GTT TAC CCA TGT GGT GCC TC	1.76	6
E	CCC TGG GTG TCC AGG AGC AAG CC		
N			
G		0.227	
F	GGC ACA TTC CGG GAC AGA GAG AA		

*M, mutated; N, normal.

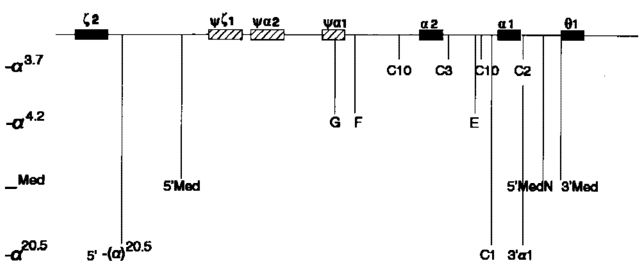

Fig. 1. Location of the oligonucleotide primers used for the identification of different α-globin gene defects by PCR. The primer nomenclature and sequence is reported in Table I. Superimposed R (for rightward) and L (for leftward) refer to the −α3.7 kb and −α4.2 kb deletion, respectively (20).

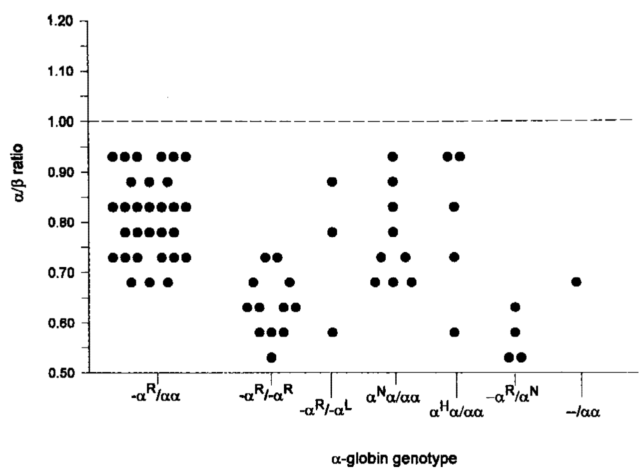

Fig. 2. Correlation between α/β globin chain synthesis ratio and α-globin genotype.

the most common (48.5% of the cases) being the heterozygous state for $-\alpha^{3.7}$ Kb deletion ($-\alpha^{3.7}/\alpha\alpha$). The homozygous condition for this defect ($-\alpha^{3.7}/-\alpha^{3.7}$) had a relative frequency of 25.5%. The ATG→ACG α_2 initiation codon nondeletion defect, detected by the *Nco*I restriction enzyme ($\alpha^N\ \alpha/\alpha\alpha$), was present in 13.6% of the subjects studied, and the α_2 IVS-1 pentanucleotide deletion, detected by the *Hph*I restriction enzyme ($\alpha^H\alpha/\alpha\alpha$), was found in 3.8% of the subjects. The other less-common genotypes globally represent about 8% of the α-thalassemia genotypes. Considering these frequencies, we developed the following strategy of PCR-based DNA

TABLE II. Relative Frequencies of Different α-Thalassemia Genotypes

Genotype	Subjects	Relative frequency (%)
$-\alpha^{3.7}/\alpha\alpha$	253	48.5
$-\alpha^{3.7}/-\alpha^{3.7}$	133	25.5
$\alpha^{N}\alpha/\alpha\alpha$	71	13.6
$\alpha^{H}\alpha/\alpha\alpha$	20	3.8
$-\alpha^{3.7}\alpha^{N}\alpha$	11	2.1
$\alpha^{N}\alpha/\alpha^{N}\alpha$	7	1.4
$--^{Med}/\alpha\alpha$	7	1.4
$-\alpha^{3.7}/-\alpha^{4.2}$	5	1.0
$-\alpha^{4.2}/\alpha\alpha$	4	0.8
$-\alpha^{20.5}\alpha\alpha$	4	0.8
$\alpha\alpha^{N}/\alpha\alpha$	2	0.4
$-\alpha^{3.7}/\alpha^{H}\alpha$	3	0.6
$\alpha^{N}\alpha/\alpha^{H}\alpha$	1	0.2

analysis to detect the most common genotypes rapidly: Using initially the oligonucleotide pairs C10-C3 and C10-C2, we are able to identify the presence of the $-\alpha^{3.7}/\alpha\alpha$ or $-\alpha^{3.7}/-\alpha^{3.7}$ genotypes, which represent 74.0% of the α-thalassemia alleles in our population. If these genotypes are absent, we obtain with the C10-C3 primers, the normal α2 gene, which can be used for digestion with *Nco*I and *Hph*I restriction enzymes. In this way, with only two steps, we are able to detect an α-thalassemia defect in more than 90% of the cases.

Hematological Phenotype–Genotype Correlation

Red blood cell (RBC) indices and HbA_2 levels are reported in Table III. When analyzed by genotype group, no significant differences were found for any of the hematological parameters among subjects with similar genotypes. Consequently, hematological data from subjects with similar genotype were considered together. For the same reason, the groups of subjects with the $-\alpha^{3.7}/\alpha\alpha$ and $-\alpha^{3.7}/-\alpha^{3.7}$ genotypes included also a few subjects with the $-\alpha^{4.2}/\alpha\alpha$ and $-\alpha^{4.2}/-\alpha^{3.7}$ genotypes, respectively. Comparison of data according to sex did not show any differences for MCV, MCH, and HbA_2, and these data were pooled. A statistically significant difference between males and females was found for Hb and RBC, which were consistently about two g/dl and 0.7×10^9/l respectively greater in males ($P < 0.05$). However, for the less common genotypes, even Hb and RBC numbers were pooled. Reduction of MCV was more evident in those subjects with the following genotypes: $\alpha^{N}\alpha/\alpha^{N}\alpha$, $-\alpha^{3.7}/\alpha^{N}\alpha$, and $--^{Med}/\alpha\alpha$. Similar results were obtained for MCH. Hemoglobin levels were mainly reduced in homozygotes for nondeletion defects. The highest RBC number was found in carriers of $--^{med}$ and $-\alpha^{20.5}/\alpha\alpha$ genotypes. There were no statistically significant differences in HbA_2 levels among the different groups of subjects.

DISCUSSION

About 10% of the Sardinian population presents microcytosis with normal HbA_2, HbF, and normal serum iron. The best-defined thalassemia disorders associated with this phenotype are heterozygous α-thalassemia, heterozygous γδβ-thalassemia, and the double heterozygous state for δ- and β-thalassemia. α-Thalassemia is by far the most common defect, at least in our population, with a frequency of about 38%, whereas double heterozygotes for δ- and β-thalassemia have been only occasionally found [8–10]. The differentiation between heterozygous α-thalassemia and the various thalassemia alleles at the β locus is a critical step in genetic counseling. In this article, we provided evidence that identification of the α-thalassemia carrier state may be fast and accurate using PCR-based methods, avoiding the cumbersome and expensive globin chain synthesis and DNA Southern blot analysis. Moreover, these latter methods, requiring the use of radioactive material, are complex and therefore available only in a few laboratories. Definition of the spectrum of α-thalassemia alleles present in our population, enabled us to design a rational strategy for a fast identification of the most common α-thalassemia defects. The same approach can be used in other Mediterranean populations since they have similar frequency and heterogeneity of α-thalassemia. The PCR-based approach made the screening for thalassemia simpler. Moreover, this strategy can be useful in couples in which both members are carriers of α-thalassemia, where definition of the genotype allows the prediction of the risk of occurrence of HbH disease and Hb Bart's hydrops fetalis syndrome. However, at least in the Sardinian population, the very low frequency of $--^{Med}$ determinant (i.e., 0.000017) results in a sporadic occurrence of HbH disease, whereas Hb Bart's hydrops fetalis syndrome is exceptional [8,17]. The diagnostic PCR-based protocol results were accurate, as demonstrated by the very low number of subjects (five of 526) whose α-thalassemia defect was not identified. In these subjects, who had a reduced α/β globin chain synthesis ratio, we have hypothesized the presence of a rare α-thalassemia mutation or of a hyperunstable α-globin variant [18,19]. α-Thalassemia in Sardinia is quite heterogeneous, with 13 different genotypes identified, the most common being the $-\alpha^{3.7}/\alpha\alpha$ and $-\alpha^{3.7}/-\alpha^{3.7}$ genotypes. Similar heterogeneity has been reported in other Mediterranean populations [7,20].

In populations where thalassemia is common, this approach may also be used to characterize those subjects with mild anemia, microcytosis, and borderline iron status, avoiding unnecessary iron treatment or repeated biochemical serum iron measurement. The above described phenotype is relatively frequent in childhood when the MCV is physiologically low, and repeated, even mild, upper respiratory tract infections may modify iron bio-

TABLE III. Hematological Data and HbA2 According to α-Globin Genotype*

Genotype	Sex	N	Hb g/dl	RBC × 10^9/l	MCV fl	MCH pg	HbA_2 (%)
$-\alpha^{3.7}/\alpha\alpha$	M	141	14.4 ± 0.9	5.7 ± 0.4	75.4 ± 4.8	25.4 ± 2.1	2.5 ± 0.3
	F	110	12.0 ± 1.0	4.8 ± 0.5			
$-\alpha^{3.7}/-\alpha^{3.7}$	M	72	13.6 ± 0.8	5.6 ± 1.0	71.3 ± 3.0	23.8 ± 2.0	2.4 ± 0.3
	F	61	11.8 ± 0.9	5.0 ± 0.4			
$\alpha^N\alpha/\alpha\alpha$	M	40	14.3 ± 1.1	5.6 ± 0.4	75.7 ± 2.8	25.5 ± 1.4	2.5 ± 0.3
	F	31	12.2 ± 0.8	4.8 ± 0.4			
$\alpha^H\alpha/\alpha\alpha$	M	10	14.4 ± 1.1	5.5 ± 0.5	76.6 ± 3.8	26.1 ± 1.4	2.5 ± 0.4
	F	10	12.3 ± 1.0	4.7 ± 0.5			
$-\alpha^{3.7}/\alpha^N\alpha$	M	6	13.3 ± 0.6	6.1 ± 0.3	66.1 ± 1.6	21.6 ± 0.7	2.5 ± 0.3
	F	5	12.1 ± 0.9	4.8 ± 0.6			
$\alpha^N\alpha/\alpha^N\alpha$	M	2	12.4 − 13.2	5.9 − 5.9	63.7 ± 2.6	21.6 ± 1.3	2.5 ± 0.2
	F	5	11.2 ± 0.7	5.2 ± 0.3			
$--^{MED}/\alpha\alpha$	M	5	13.9 ± 1.3	6.6 ± 0.6	65.0 ± 3.3	21.0 ± 1.3	2.4 ± 0.1
	F	2	12.4 ± 11.1	5.6 ± 5.5			
$-\alpha^{3.7}/-\alpha^{4.2}$	M	1	13.0	5.7	67.2 ± 3.5	22.5 ± 1.6	2.3 ± 0.2
	F	3	10.6 ± 1.30	4.8 ± 0.3			
$-\alpha^{4.2}/\alpha\alpha$	M	1	14.7	5.8	77.5	25.3	2.5
	F	1	11.3	5.0	64.1	20.4	2.4
$-\alpha^{20.5}/\alpha\alpha$	M	3	12.0 ± 1.3	6.1 ± 0.5	67.8 ± 0.9	21.0 ± 1.0	2.3 ± 0.7
	F	1	10.0	4.75			
$\alpha\alpha^N/\alpha\alpha$	F	2	12.6 − 11.0	4.9 − 4.2	79.0 − 77.6	25.5 − 26.2	2.4 − 2.5
$-\alpha^{3.7}/\alpha^H\alpha$	M	1	12.0	5.8	63.7 ± 2.9	20.8 ± 1.0	2.3
	F	2	11.2 ± 10.6	5.1 ± 5.3			2.2 − 2.4
$\alpha^N\alpha/\alpha^H\alpha$	F	1	11.0	5.5	59.7	19.9	2.7

*Hb, hemoglobin; N, number; RNC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

chemical determination, making the evaluation of the hematological indices less reliable.

The hematological phenotype of carriers of different α-thalassemia genotypes is heterogeneous and there is a wide overlap between groups. Subjects homozygous for the α2 globin gene nondeletion defect ($\alpha^N\alpha/\alpha^N\alpha$) or with the $\alpha^H\alpha/\alpha^N\alpha$ genotype show the most severe phenotype with moderate microcytic hypochromic anemia. Carriers of $--^{Med}/\alpha\alpha$ and $-\alpha^{20.5}/\alpha\alpha$ defects have only a mild reduction of Hb levels because of a compensatory increase in the number of red cells. The hematological phenotype of $-\alpha^{3.7}/\alpha\alpha$ heterozygotes is not representative of the real phenotype of these carriers. In fact, we selected only patients with reduced MCV and MCH, but we know that the $-\alpha^{3.7}/\alpha\alpha$ genotype is usually silent and most of the $-\alpha^{3.7}/\alpha\alpha$ carriers have RBC indices in the normal range and are therefore not included in this group.

Coexistence of α-thalassemia and the other phenotypically similar genotype, that is heterozygous δ- and β-thalassemia in the same subject, is rare in our population; in fact only three cases have been found so far. In these cases, HbA_2 is reduced to borderline levels (i.e., 3.2–3.6%) and does not reach normal values. Therefore, we consider hemoglobin A_2 the most useful parameter to suspect the presence of β-thalassemia in combination with δ- and α-thalassemia.

In conclusion, the application of simple and rapid PCR-based methods may have major implications for screening and for hematological diagnosis in populations where α-thalassemia is common.

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